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ORIGINAL RESEARCH PAPER

Cloning of milk-derived bioactive peptides in *Streptococcus thermophilus*

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Abstract The enzymatic breakdown of milk proteins releases bioactive peptides. Two such peptides are the 11-residue antimicrobial peptide from bovine lactoferrin (BL-11) and the 12-residue hypotensive peptide from α_{s1}-casein (C-12). These two peptides have now been cloned in *Streptococcus thermophilus* to develop strains that enhance the functionality and nutritional value of dairy food products. Nucleic acid sequences encoding the peptides were generated by overlapping PCR and were subsequently cloned into a new expression vector under control of the ST_{2201} promoter. *S. thermophilus* transformants were successfully identified using GFP as a selectable marker. The presence of the synthetic gene constructs in *S. thermophilus* was confirmed by PCR.

Keywords Bioactive peptides · Lactoferricin · Hypotensive peptide · *Streptococcus thermophilus*

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Introduction

Bovine milk contains a large number of bioactive peptides with various biological activities that may impact human health. Several bioactive peptides with antimicrobial, immunomodulating and hypotensive activities are built into the primary structures of milk proteins and are released by enzymatic hydrolysis during digestion or by microbial activity during the manufacturing of fermented dairy products (Clare and Swaisgood 2000; LeBlanc et al. 2002). Because of their potential as physiological modulators, there is an interest in milk-based bioactive peptides as ingredients in functional foods with enhanced nutritional and health-promoting properties.

The iron-chelating protein, lactoferrin, contains a 25-amino acid residue peptide, lactoferricin, near its *N*-terminus that may be liberated by peptic digestion (Bellamy et al. 1992a). Lactoferricin has more potent antimicrobial activity than undigested bovine lactoferrin (Tomita et al. 1991) and is active against a diverse range of Gram-positive and Gram-negative bacteria including, but not limited to, *Listeria monocytogenes*, *Staphylococcus aureus*, *Campylobacter jejuni, Escherichia coli* and *Salmonella enteritidis* (Bellamy et al. 1992b). Further studies demonstrated that an 11-amino acid residue peptide (BL-11) (RRWQWRMKKLG) within lactoferricin retained broad spectrum antimicrobial activity while displaying significantly lower hemolytic activity

(Kang et al. 1996), which increased its potential for therapeutic applications.

Tryptic digestion of bovine milk casein results in the release of peptides that possess hypotensive activity (Maruyama et al. 1987; Tauzin et al. 2002). One such peptide consists of 12-amino acid residues (C-12) and is found in the *N*-terminus of α_{s1}-casein (FFVAPFPEVFGK). The C-12 peptide inhibits the angiotensin I-converting enzyme (ACE), which hydrolyzes angiotensin I to the potent vasoconstrictor, angiotensin II (Skeggs et al. 1956). The C-12 peptide can lower blood pressure in spontaneously hypertensive rats (Karaki et al. 1990) and in humans (Townsend et al. 2004), thus demonstrating its potential as a functional-food ingredient.

Although the amount of bioactive peptides available in milk is limited by protein concentration, the production of larger amounts needed for food supplementation may be possible by using food-grade dairy fermentation bacteria as peptide production systems. This report summarizes the results of research on the development of synthetic genes for the delivery and expression of the BL-11 antimicrobial and C-12 hypotensive peptides in *Streptococcus thermophilus* which is an essential dairy bacterium used in commercial production of cheeses as well as yogurt. Since *S. thermophilus* possesses several types of peptidase systems for generating free amino acids from oligopeptides following the proteolytic breakdown of caseins (Savijoki et al. 2006), we also tested the stability of BL-11 and C-12 peptides in the presence of *S. thermophilus* cell suspensions, simulating conditions of yogurt fermentation.

Materials and methods

Bacterial strains and growth media

The targeted plasmid-free host *Streptococcus thermophilus* ST128 was grown in TYL broth at 37°C without agitation (Somkuti and Steinberg 1986). The sensitivity of ST128 to the BL-11 antimicrobial peptide was tested by the agar-well diffusion method (Tagg and McGiven 1971). Genetic transformants of ST128 were propagated in TYL medium at 32°C. *Escherichia coli* strains DH5α and DM1 (*dam*$^{-}$) were used as hosts for subcloning plasmids. *E. coli* transformants were grown in BHI medium (BD Diagnostics, Franklin Lakes, NJ) supplemented with 100 μg ampicillin ml^{-1} at 32 or 37°C with vigorous agitation.

Resting cell assays

The BL-11 antimicrobial and C-12 hypotensive peptides were synthesized by EZBiolab Inc. (Westfield, IN). The sensitivity of the peptides to cell-bound peptidases was tested using resting cells of ST128, prepared from a mid-growth phase (OD_{660} = 0.5) culture. Cells were collected by centrifugation and washed twice in 10 mM phosphate buffer (pH 4.5). Washed cells were concentrated 20-fold in 10 mM phosphate buffer. Heat-killed (boiled) cells were used as the controls. Live cell suspensions were incubated with 5 μg of either the 11-residue (BL-11) or 12-residue (C-12) synthetic peptides at 37°C for 120 min, followed by boiling for 10 min to inactivate enzyme activity. After cooling, the reaction mixtures were run on a NuPAGE 12% Bis–Tris gel (Invitrogen, Carlsbad, CA) and visualized with Simply Blue Safe Stain (Invitrogen).

Construction of synthetic genes

Nucleic acid sequences corresponding to the BL-11 lactoferricin-derived peptide and the C-12 α_{s1}-casein-derived peptide were generated with a bias towards streptococcal codon usage (Malke 1986). Synthetic genes encoding each peptide were constructed using an overlapping polymerase chain reaction (PCR) technique (Dillon and Rosen 1990). The sequences of the DNA oligonucleotides used as primers for PCR reactions were synthesized by Integrated DNA Technologies (Coralville, IA) (Supplementary Table 1). Oligonucleotides were designed for the 11-mer (11N and 11C) and 12-mer (12N and 12C) peptides that contained 21 nucleotides of overlapping sequence. The first PCR (15 cycles: 95°C for 30 s, 55°C for 30 s, 74°C for 30 s) allowed the two oligonucleotides to ligate and generate the full-length products using Taq polymerase (New England Biolabs, Ipswich, MA). A second PCR (30 cycles: 95°C for 30 s, 50°C for 30 s, 74°C for 30 s) was performed using primers specific for the 11-mer (11F and 11R) or 12-mer (12F and 12R) synthetic genes to amplify

the final product for cloning. The forward primers (11F and 12F) contained a *Bcl*I restriction site and the reverse primers (11R and 12R) contained a *Xba*I restriction site for directional cloning.

DNA cloning procedures

The green fluorescent protein (GFP) expression vector pG341Pa was constructed according to a strategy previously described (Somkuti and Steinberg 1999). Oligonucleotides were designed to amplify the *S. thermophilus* chromosomal promoter ST_{2201} (Somkuti and Solaiman 1997). The forward primer (2201F) contained a *Sac*I recognition sequence and the reverse primer (2201R) contained both *Bcl*I and *Sac*I recognition sequences. The amplified ST_{2201} PCR product was inserted into pG341Pa at the *Sac*I sites present in the vector. Cloning steps involving the use of *Sac*I required the propagation of the plasmid in *E. coli* DM1 to prevent methylation of the restriction site. Oligonucleotides 2201A and 2201B were used to determine the orientation of the insert in pG341Pa. The *Bcl*I site from the 2201R primer and a unique *Xba*I site in pG341Pa were used for insertion of the bioactive milk peptides downstream of the ST_{2201} promoter. Plasmids were isolated from *E. coli* by the alkaline lysis method (Ausubel et al. 1987) and further purified by CsCl/ethidium bromide ultracentrifugation (Stougaard and Molin 1983). PCR products used in cloning were purified with the QIAquick PCR purification kit (Qiagen Inc, Valencia, CA). DNA in plasmid preparation was estimated using the extinction coefficient of $E^{1\%}_{260} = 200$. Restriction endonucleases, *Taq* polymerase and T4 DNA ligase were used according to the vendor's instructions (NE Biolabs). Gel electrophoresis was performed in TAE buffer (0.04 M Tris, 0.02 M acetic acid, 0.001 M EDTA, pH 8.0) with 1% agarose or 0.8% low-melting point agarose. DNA fragments required for ligations were recovered from low-melting point agarose gels (Wieslander 1979).

Transformation of freshly prepared *E. coli* competent cells was carried out by a heat-shock method (Sambrook et al. 1989), while *S. thermophilus* ST128 was electrotransformed by a standard protocol previously described (Somkuti and Steinberg 1988). Green fluorescent transformants of *E. coli* were scored on BHI agar containing ampicillin after 24 h incubation at 32°C, under UV illumination (365 nm). *S. thermophilus* electrotransformants were scored on antibiotic-free TYL agar after incubation for 48 h at 32°C.

DNA sequencing

PCR products were cleaned with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Nucleic acid sequencing was performed using an ABI PRISM 3730 (Perkin-Elmer, Wellesley, MA) DNA analyzer with ABI PRISM Big Dye terminator cycle sequencing reagent and oligonucliotide primers 2201F and 2201B. Obtained sequences were analyzed using Sequencher 4.2 (Gene Codes Corp., Ann Arbor, MI).

RT-PCR analysis

Streptococcus thermophilus colonies which fluoresced under UV light were used to inoculate 5 ml of TYL broth. Cultures were initially grown overnight and then diluted into fresh TYL broth and grown to mid-exponential phase ($OD_{660} = 0.4$–0.5), at which point the cells were collected by centrifugation. Total RNA was isolated from *S. thermophilus* using the RiboPure-Bacteria kit (Ambion, Austin, TX). Reverse transcriptase PCR (RT-PCR) was performed using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA polymerase (Invitrogen). A control reaction was performed in the absence of RT using Platinum *Pfx* DNA polymerase (Invitrogen) to ensure the RNA prep was not contaminated with trace amounts of DNA. Oligonucleotide primers 12F and 12R were used for amplification. EubA and EubB primers (Cottrell and Kirchman 2000) for amplification of the 16S rRNA genes were used as a control.

Results and discussion

Host sensitivity and stability of synthetic peptides

The targeted *S. thermophilus* ST128 host strain was not inhibited by the BL-11 synthetic antimicrobial peptide when tested at concentrations ranging from 0 to 5 mg ml^{-1}. However, *S. thermophilus* is equipped

with a number of intracellular peptidases which generate free amino acids from oligopeptides produced from milk proteins by cell-envelope protease and allow the bacteria to utilize them as an energy source for growth (Hols et al. 2005; Savijoki et al. 2006). This raised concerns about the integrity of BL-11 and C-12 peptides produced in situ by recombinant *S. thermophilus* in fermented dairy products following exposure to the host's internal peptidase systems. Therefore, the stability of synthetic 11- and 12-residue peptides was tested in the presence of mid-growth phase ST 128 cells (Fig. 1). The cells were collected and resuspended in phosphate buffer at pH 4.5, which is characteristic of the level of acidity reached in yogurt fermentations. The results of SDS-PAGE analysis showed that after incubation in the presence of live resting cells for 120 min, both peptides apparently remained intact (Fig. 1). This implied that recombinant BL-11 and C-12 peptides produced in situ may be expected to persist in fermented dairy products.

Construction of expression vectors and synthetic genes

The outline for the construction of the expression vectors pRS1 and pRS2 is shown in Fig. 2. The first stage of construction involved replacing the erythromycin resistance gene from pG341Pa with the *S. thermophilus* chromosomal promoter ST_{2201}. The vector pG341Pa was digested with *Sac*I releasing a 0.9 kb fragment containing the Erm resistance gene. The ST_{P2201} promoter and ribosome binding site (RBS) were amplified by PCR and the resulting product (83 bp) was cloned into pG341Pa at the exposed *Sac*I sites. Orientation of the insert was confirmed by PCR. The resulting vector contained the ST_{2201} promoter with unique *Bcl*I and *Xba*I restriction sequences located downstream of the RBS. These restriction sites allow for subsequent cloning of peptides or proteins in *S. thermophilus*. Heterologous expression of peptides or proteins in *E. coli* hosts should also be possible since it was previously shown that the ST_{P2201} promoter is fully functional in *E. coli* (Somkuti and Solaiman 1997).

The second stage required construction and subsequent cloning of the synthetic genes encoding the BL-11 and C-12 bioactive peptides. Nucleic acid sequences encoding the two peptides were generated with a codon usage table for streptococci (Malke

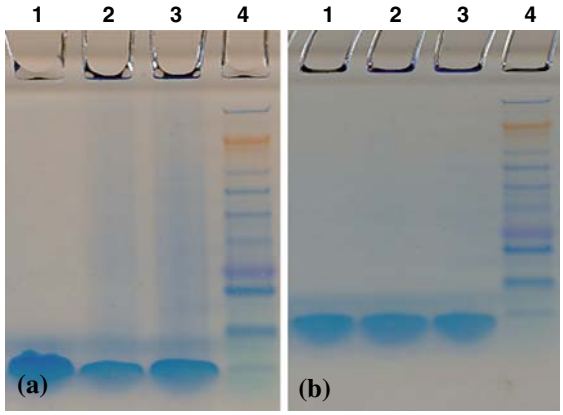

Fig. 1 Stability of the BL-11 (**a**) and C-12 (**b**) peptides in the presence of mid-exponential *S. thermophilus* ST128 cells. Lane 1: synthetic peptide preparations, Lane 2: synthetic peptide incubated with live *S. thermophilus* ST128 resting cells, Lane 3: synthetic peptides incubated with boiled *S. thermophilus* ST128 cells, Lane 4: molecular weight marker

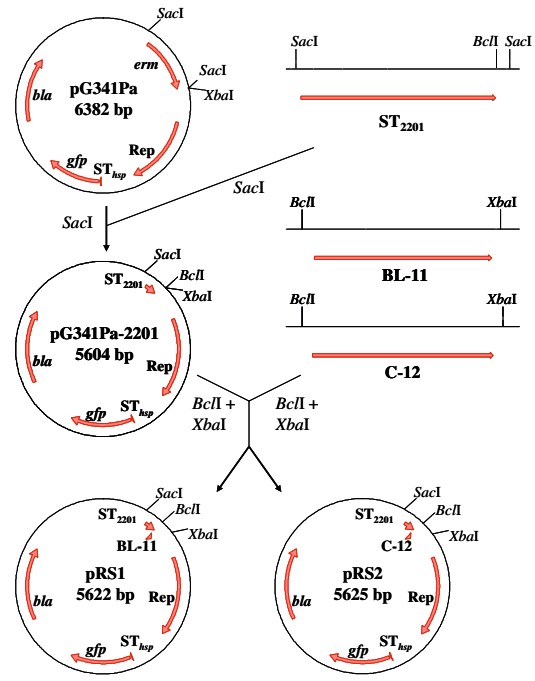

Fig. 2 Outline of the construction of expression vectors pRS1 and pRS2. *erm*: Erythromycin resistance gene, *bla*: Ampicillin resistance gene, ST_{hsp}: *S. thermophilus hsp* promoter, *gfp*: green fluorescence protein, ST_{2201}: *S. thermophilus* 2201 promoter, BL-11: 11 residue lactoferricin peptide, C-12: 12-residue antihypertensive peptide

1986) to facilitate optimal expression in *S. thermophilus*. The nucleic acid sequences for the synthetic genes were also designed to include a codon for methionine (atg) at their 5′-end and a stop codon (taa) at their 3′-end to allow for the proper initiation and termination of translation. The synthetic genes were constructed by the overlapping PCR technique with the final products being 57 bp and 60 bp corresponding to the BL-11 and C-12 peptides, respectively (Fig. 3). The synthetic genes contained a 5′-*Bcl*I site and a 3′-*Xba*I and were cloned into the new expression vector at the same unique sites, resulting in the formation of vectors pRS1 (BL-11) and pRS2 (C-12) (Fig. 1). The new plasmid constructs were confirmed by sequencing PCR products which included the cloned promoter and synthetic genes (Fig. 4). The synthetic genes were not constructed with signal peptides since it is unclear if *S. thermophilus* possesses the required proteins for processing and export of propeptides. *S. thermophilus* has not been shown to express endogenous bacteriocins which may require such processing and sequence analysis of other *S. thermophilus* strains has indicated that some components of the Sec translocase may be absent from the genome (Hols et al. 2005). In addition, an increasing number of secreted peptides, which are not synthesized with *N*-terminal signal sequences, have been identified in lactic acid bacteria and are thought to be exported by ABC transporters or multidrug resistance transporters (Cintas et al. 2000; Gajic et al. 2003).

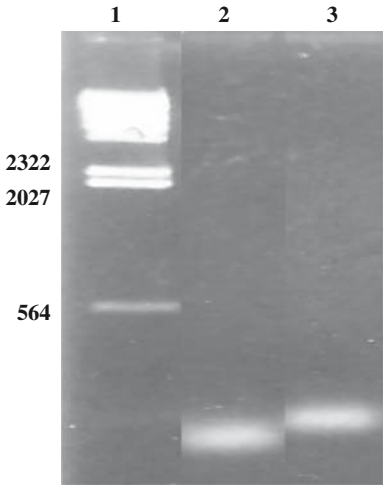

Fig. 3 Synthetic gene products from overlapping PCR. Lane 1: lambda *Hin*dIII digest, Lane 2: synthetic gene encoding BL-11 peptide (57 bp), Lane 3: synthetic gene encoding C-12 peptide (60 bp). Numbers to the left of image are in base pairs

Previous work in our laboratory showed the usefulness of GFP as a selection marker controlled by the ST_{2201} promoter in the construction of insertion-inactivation shuttle vectors for cloning work in *E. coli* and *S. thermophilus* (Solaiman and Somkuti 1997). Subsequent work on GFP expression under the control of the ST_{hsp} in *S. thermophilus* transformants containing pG341Pa also confirmed the value of GFP as a selectable marker in food-grade bacteria (Somkuti and Steinberg 1999). The pRS vectors described here are

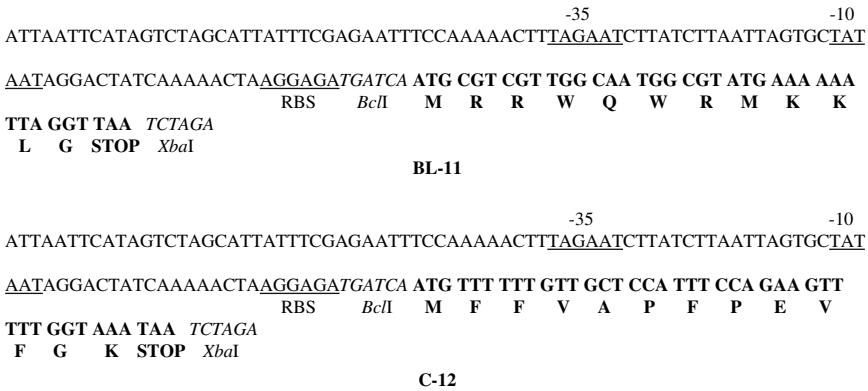

Fig. 4 Sequence analysis of cloned peptides in pRS1 and pRS2. The forward sequencing primer was located 27 bp upstream of the ST_{2201} promoter and the reverse primer was located 361 bp downstream of the cloned peptide resulting in a 561 bp (BL-11) or a 564 bp (C-12) PCR product. The nucleotides and amino acids encoding the BL-11 and C-12 peptides are shown in bold. The −35 and −10 regions of the ST_{2201} promoter and the RBS are underlined. Restriction endonuclease recognition sequences are italicized

unique in that they do not contain an antibiotic resistance marker gene that would be expressed in *S. thermophilus*. Since plasmids containing antibiotic resistance genes are not considered "food-grade" vectors, the Erm resistance gene was excised from pG341Pa and the identification of *S. thermophilus* transformants was based solely on GFP expression which resulted in fluorescent colonies. Preliminary work with Long-Evans Hooded rats indicated that GFP has a low allergenicity and is not likely to represent a health risk (Richards et al. 2003), suggesting it may become an acceptable genetic tool in generally recognized as safe (GRAS) bacteria used during food production. Although in this work the Erm gene was removed from pRS vectors, the Amp resistance gene was left intact in order to facilitate the cloning and detection of the expression vectors in *E. coli*.

Screening of *S. thermophilus* electrotransformants

The expression vectors were maintained in *E. coli* DH5α under Amp selection. As previously reported, the ST_{hsp} promoter was active in *E. coli* (Somkuti and Steinberg 1999) as 100% or the Amp resistant colonies fluoresced under UV illumination when grown at 32°C. Growth of the *E. coli* transformants on agar or in broth was not altered due to the presence of the pRS plasmids suggesting the cloned peptides were not toxic to the cells.

Plasmids isolated from *E. coli* were further purified by CsCl/ethidium bromide gradient ultracentrifugation before electroporation into *S. thermophilus* ST128. Following purification the plasmid concentrations were 0.66 μg μl^{-1} for pRS1 (BL-11) and 0.77 μg μl^{-1} pRS2 (C-12). Electrotransformants were grown on TYL agar for 48 h at 32°C to allow for GFP expression. Potential electrotransformants were screened with UV illumination to identify green fluorescent colonies. For pRS1, at least 5,000 potential electrotransformants were screened and 2 fluorescent colonies were identified, giving a transformation efficiency of 4×10^{-5} c.f.u μg^{-1} of plasmid DNA. For pRS2, over 27,000 electrotransformants were screened and 3 fluorescent colonies were identified giving an estimated transformation efficiency of 1.1×10^{-5} c.f.u μg^{-1} of plasmid DNA. The transformation efficiencies obtained were markedly lower than

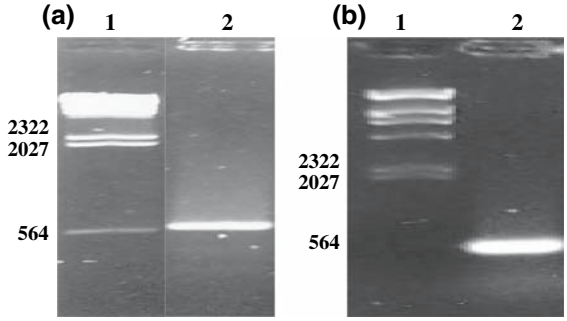

Fig. 5 PCR products confirming the presence of the synthetic genes encoding the BL-11 and C-12 peptides in *S. thermophilus* ST128. Primers specific to the vector were used to amplify the segment of pRS1 (**a**) and pRS2 (**b**) that contained the ST_{2201} promoter and the cloned gene. Bands corresponding to 561 bp for pRS1 (**a**: lane 2) and 564 bp for pRS2 (**b**: lane 2) were observed confirming the presence of the synthetic genes. Lane 1 in both gels contains a lambda *Hin*dIII digest. Numbers to the left of image are in base pairs

that previously reported for *S. thermophilus* ST128 (Somkuti and Steinberg 1988) but the results demonstrated that in the absence of antibiotic selection, GFP can be used successfully as a selectable marker for identifying genetic transformants.

The electrotransformants fluorescing under UV illumination were further screened by PCR to confirm the presence of the synthetic genes in pRS1 and pRS2 (Fig. 5). The PCR performed with the primer set 2201A and 2201B resulted in bands of 561 bp and 564 bp for pRS1 and pRS2 respectively, confirming the presence of the cloned BL-11 and C-12 peptides.

Plasmid stability

The vectors pRS1 and pRS2 were stably maintained in *E. coli* DH5α. Fluorescent colonies were used to inoculate BHI broth free of Amp and incubated overnight at 32°C. Subsequent plating of the broth cultures on BHI agar without Amp resulted in 100% fluorescent colonies. Similar results were observed when the transformed *E. coli* cells were subcultured a second time in BHI broth before plating, suggesting the pRS plasmids are stable in *E. coli* in the absence of antibiotic selection (data not shown).

The pRS vectors have been maintained in *S. thermophilus* for over a year in the absence of antibiotic pressure by continuous passage on TYL

agar, with close to 100% fluorescing colonies. Initially, following overnight growth in TYL broth, a maximum of 5% fluorescing colonies were recovered on TYL agar. However, the continuous passage of electrotransformants on TYL agar led to the selection of more stable strains. Up to 97% of electrotransformants containing pRS2 remained fluorescent following 3 passages in TYL broth at 34 or 37°C and up to 50% maintain the plasmid after 5 passages in TYL broth. We are currently screening for *S. thermophilus* strains that maintain the pRS1 plasmid at a similar frequency as observed for pRS2. Currently up to 91% of the electrotransformants maintain pRS1 after 1 passage in TYL broth and up to 60% maintain the vector for 2 passages.

Peptide expression

Streptococcus thermophilus containing pRS2 were tested for their ability to express the C-12 peptide in TYL broth. Exponentially growing cells were used for RT-PCR analysis after 2 passages in broth medium (Fig. 6). Lane 1 shows the 60 bp band expected for the C-12 peptide and Lane 2 shows amplification of the 16S rRNA (1.5 kb). The absence of a band in Lane 3 where only DNA polymerase was present confirmed that the RNA preparation was not contaminated by trace amounts of DNA. Work is in progress on selecting additional *S. thermophilus* host strains for stabilizing the expression of the BL-11 antimicrobial peptide.

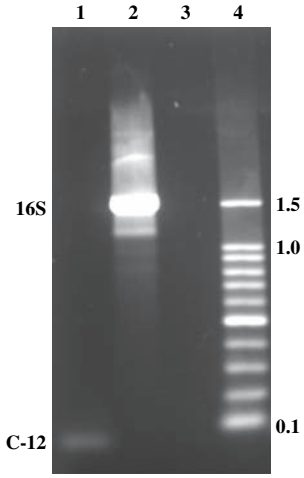

Fig. 6 C-12 peptide expression measured by RT-PCR. Lane 1: amplified cDNA of C-12 peptide, Lane 2: positive control, amplified cDNA of 16S rRNA, Lane 3: negative control, amplification of 16S rRNA gene with Pfx DNA polymerase, Lane 4: 100 bp molecular weight marker

Conclusions

To our knowledge this is the first report describing the cloning and expression of synthetic genes in lactic fermentation bacteria that encode mature bioactive peptides which are normally derived from bovine milk proteins. In addition, the results confirmed that GFP expression may be used as a selectable marker for the identification of *S. thermophilus* transformants for food applications. Work continues to optimize the expression of these peptides, including the use of leader sequences and cloned proteins required for processing of these sequences to demonstrate biological activity. By genetically engineering *S. thermophilus* to produce these peptides we hope to increase their availability, potentially improving the functionality and nutritional value of fermented dairy and other food products.

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